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Calcium ions enhance systemin activity and play an integral role in the wound response

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Abstract

The activation of defense genes in response to wounding involves the integration of multiple inputs from various signaling molecules produced in different tissues and cellular compartments. In this report, we demonstrate that calcium and magnesium ions enhance systemin activity and that calcium fluxes are necessary for wound gene activation. During wounding, intracellular contents such as calcium, magnesium and the defense peptide systemin, a potent activator of the wound response, are released into the apoplastic space. Our analysis showed that elevated concentrations of calcium or magnesium ions in the apoplast significantly enhanced the biological activity of systemin by decreasing the concentration necessary to induce maximal proteinase inhibitor accumulation by 100-fold. Calcium ionophores disrupt calcium gradients across biological membranes and consequently elevate intracellular calcium levels. Plants treated with the calcium ionophores ionomycin and A23187 induced the accumulation of proteinase inhibitor proteins to levels similar to those produced by wounding. Ionophore-mediated proteinase inhibitor induction was blocked in the jasmonic acid signaling mutant *def-1*, indicating that increased levels of intracellular calcium activated the octadecanoid pathway leading to wound gene activation. Calcium transport antagonists (nifedipine, verapamil, ruthenium red, and erythrosine-B) were found to inhibit proteinase inhibitor synthesis in response to wounding, whereas extracellular calcium chelators were found to induce the synthesis of proteinase inhibitors in a jasmonic acid-dependent and systemin-independent manner. Taken together, our data show that calcium and potentially magnesium ions play an integral role in mediating the plant's response to wounding.

Keywords: Calcium; Divalent cations; Magnesium; Proteinase inhibitors; Systemin; Wound response

1. Introduction

Solanaceous plants respond to mechanical wounding or herbivorous insect attack by inducing the synthesis of a wide array of defense related proteins at the wound site and systemically throughout the plant [1–3]. Proteinase inhibitor synthesis is a well-characterized marker of the tomato wound response [4]. Proteinase inhibitors are anti-digestive proteins that interfere with proteases in the intestines of insects depriving them of essential amino acids [5]. Wounding causes the release and mobilization of an 18-amino acid polypeptide called systemin [6] and other defense-related peptides [7–9]. The binding of systemin to the cell surface receptor kinase SR160 [10] activates a wide range of physiological and biochemical processes including ion transport, membrane depolarization and alkalinization of the apoplast or growth medium [11–13], the formation of hydrogen peroxide [14], the activation of mitogen-activated protein kinases [15–16], an increase in intracellular calcium (Ca^{2+}) concentrations [17–18], activation of calmodulin [19], and a phospholipase A_2 [20-21]. The phospholipase acts on plant membranes to release linolenic acid, which is subsequently converted to biologically active oxylipins, including 12-oxo-phytodienoic acid and jasmonic acid (JA), via the octadecanoid pathway. These signaling

Abbreviations: BAPTA, O,O′-Bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ca^{2+}, calcium; CBL, calcineurin B-like Ca^{2+} binding proteins; EB, erythrosine-B; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; ER, endoplasmic reticulum; JA, jasmonic acid; Mg^{2+}, magnesium; Mn^{2+}, manganese; MJ, methyl jasmonate; Nif, Nifedipine; Inh I, proteinase inhibitor I; Inh II, proteinase inhibitor II; PM, plasma membrane; RR, ruthenium red; Ver, Verapamil

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molecules, as well as others coupled with response pathways that are independent of JA, lead to the localized and systemic wound response (reviewed in Refs. [2,3,22]).

Ca^{2+} appears to be a ubiquitous signal in all plant tissues, and plays key regulatory roles in response to environmental or developmental stimuli. In the resting state Ca^{2+} levels are high in the apoplast, mitochondria, vacuole and ER (~1 mM), and low in the cytosol (~0.0001 mM). In response to various stimuli, Ca^{2+} ions are released into the cytosol via channel proteins and pumped back into the organelles and apoplast via Ca^{2+} pumping ATPases (see reviews [23–25]). Ca^{2+}-binding proteins serve as primary regulators of internal Ca^{2+} levels in plant cells. Ca^{2+}-binding proteins, such as calmodulin or calcium-dependent protein kinases, function to buffer intracellular Ca^{2+} levels or translate intracellular oscillations of free Ca^{2+} levels into signal-specific cellular responses [26–29].

The specific biological role of Ca^{2+} in the wound response signaling cascade is unclear. Increases in cytosolic Ca^{2+} levels have been shown to be triggered by the wound signaling peptide, systemin, resulting from an influx of extracellular Ca^{2+} through several types of Ca^{2+} permeable channels and mobilization of internal Ca^{2+} stores [17]. However, this research failed to link these increases in calcium concentration with activation of wound responsive genes. Increases in cytosolic Ca^{2+} levels were also shown to activate a Ca^{2+}-dependent protein kinase, which may in turn phosphorylate and inhibit the plasma membrane H^+-ATPase, resulting in alkalinization of the extracellular matrix and depolarization of the plasma membrane [11–13,30]. In addition, transcript levels of calmodulin increased in response to systemin and wounding suggesting that binding of calcium to calmodulin might be an integral part of the systemin/wound signaling pathway [19,29,31].

In this report, we have investigated the effects of divalent cations on the wound response in tomato plants. Elevated concentrations of magnesium (Mg^{2+}) and Ca^{2+} in the appoplast were shown to significantly enhance the biological activity of systemin. Ca^{2+} ionophores were found to induce the accumulation of proteinase inhibitors, while Ca^{2+} antagonists were found to inhibit the accumulation of proteinase inhibitors resulting from wounding. This is the first report that directly correlates calcium fluxes and wound gene activation. We discuss potential roles for calcium and magnesium ions in the wound response, which provides further insight into the mechanisms that regulate plant defense gene expression.

2. Materials and methods

2.1. Plant material

Wild type (*Lycopersicon esculentum* cv Castlemart and cv Better Boy); mutants *def-1* and *spr-1* (cv Castlemart); and transgenic tomato plants over-expressing prosystemin cDNA in antisense orientation (cv Better Boy) were grown for 14–15 days in peat pots for 17 h at 28 °C under >300 $\mu E\ m^{-2}\ s^{-1}$ light followed by a 7 h, 17 °C dark period. Plants at this stage of development displayed two expanded leaves and a small apical leaf.

2.2. Plant treatments and proteinase inhibitor bioassay

To assay proteinase inducing activity of test compounds, feeding assays were performed 14–15 day old plants were excised at their base of the stem with a razor blade and supplied through their cut stems with the following solutions: water, 10 mM phosphate buffer (pH 6.8), systemin in water or phosphate buffer, different concentrations of NaCl, chelators (ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid [EGTA] pH 8.0; ethylenediaminetetraacetic acid [EDTA] pH 8.0; citric acid pH 8.36; O,O′-Bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid [BAPTA] pH 7.0) or calcium ionophores (A23187 or ionomycin) in 1.7 mL microfuge tubes (2 plants/tube). The plants were incubated in closed plexiglass boxes for 1–1.5 h (fluid uptake ranged between 60 and 90 μL/h) and then were transferred to vials containing distilled water. After treatment some plants were wounded 1–2 times on both leaves with a hemostat perpendicular to the midvein. All plants were then placed back into sealed plexiglass boxes containing an open jar of 10 N NaOH as a CO_2 trap, and incubated for 24 h under constant light. Leaf juice from individual plants was expressed and proteinase inhibitor concentrations were determined using a radial immunodiffusion assay as previously described [32].

To examine the effects of calcium antagonists, excised plants were supplied with either 10 μM ruthenium red, 10 μM erythosine-B, 10 μM nifedipine or 10 μM verapamil in 10 mM phosphate buffer through their cut stem for 1–2 h prior to wounding or prior to supplying systemin or water for an additional 2 h. After treatments, the plants were transferred to water, incubated for 24 h under the same condition described above and subsequently assayed for proteinase inhibitor content.

In experiments involving treatment with methyl jasmonate (MJ), excised plants were supplied through their cut stem with either 10 μM ruthenium red or 10 μM erythosine-B, in 10 mM phosphate buffer for 1 h, then transferred to vials of distilled water. These plants were then exposed to MJ vapor for 24 h (1 μL stock of MJ was diluted in 25 μL of ethanol and the total volume was applied to a cotton wick inside a sealed plexiglass box) and assayed for proteinase inhibitor content.

3. Results

3.1. Ca^{2+} ionophores induce proteinase inhibitor accumulation in plants

Ca^{2+} ionophores are used diagnostically in many biological systems to study cellular responses to elevations in the concentration of intracellular Ca^{2+} [25,33–37]. Excised tomato seedlings treated with the Ca^{2+} ionophore ionomycin exhibited a dose-dependent induction of the serine proteinase inhibitors I and II (Inh I and Inh II, respectively) (Fig. 1A), similar to the induction produced by mechanical wounding. Ionomycin had no effect at 0.1 μM, was maximally effective at 1.0 μM, and exhibited reduced activity at a concentration of 10 μM. Another well-documented calcium ionophore, A23187, also

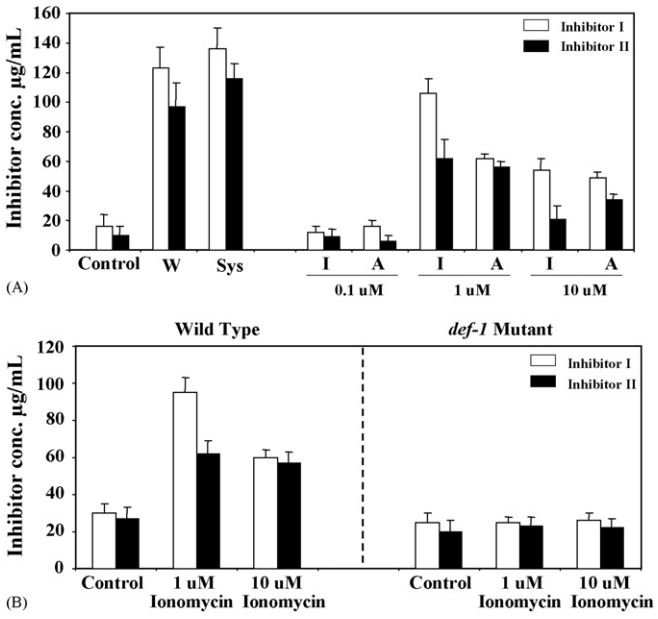

Fig. 1. Proteinase inhibitor induction by the calcium ionophores. (1A) Young tomato plants were supplied through their cut stems with the indicated concentrations of ionomycin (I) or A23187 (A) for 1 h and then transferred to water. Control plants were supplied with buffer alone (control), 2.5 pmol of systemin (SYS) or incubated with buffer for 1 h and subsequently wounded on the leaves and then transferred to water. (2B) Proteinase inhibitor induction by the calcium ionophore ionomycin in the tomato mutant *def-1*. Proteinase inhibitor accumulation in leaves was measured 24 h after treatment. All solutions were supplied to plants in 10 mM phosphate buffer, pH 6.8. Data represent the mean ± S.D. of four independent experiments (six plants/treatment).

induced proteinase inhibitors when supplied at concentrations between 1 and 10 μM, but the levels of inhibitor induction were only 70% of those observed using ionomycin at similar concentrations (Fig. 1A). These experiments indicate that ionophore-mediated increases in intracellular levels of free Ca^{2+} induce proteinase inhibitor synthesis.

In order to determine whether Ca^{2+} ionophore-mediated induction of proteinase inhibitors was jasmonic acid-dependent, the tomato mutant *def-1* was supplied with ionomycin. The *def-1* mutant has been shown to have a block in the octadecanoid pathway and a severely reduced wound response [38–39]. After treatment with ionomycin the *def-1* mutant displayed no significant induction of proteinase inhibitors (Fig. 1B) indicating that increased levels of intracellular calcium leads to activation of the octadecanoid pathway resulting in wound gene induction.

3.2. Ca^{2+} transport antagonists differentially inhibit proteinase inhibitor induction by wounding

The induction of the wound response by ionomycin suggested that elevations in intracellular Ca^{2+} levels contributed to the observed increases in proteinase inhibitor accumulation. Therefore, different Ca^{2+} antagonists were assayed for their effects on proteinase inhibitor accumulation in response to wounding. Verapamil (Ver) and Nifedipine (Nif) have been shown to block L-type calcium channels in the plasma membranes of animal cells, whereas ruthenium red (RR) and erythrosine-B (EB) are generally thought to inhibit the mobilization of Ca^{2+} from internal stores. Moreover, EB has been shown to inhibit P-type plasma membrane Ca^{2+}-ATPase. Fig. 2 shows that pre-treatment of excised tomato seedlings with 10 μM of RR, EB, Nif or Ver for 1–2 h prior to wounding resulted in reduced proteinase inhibitor accumulation. Pretreating plants with Ver or Nif prior to wounding resulted in a 30–50% decrease in the levels of proteinase inhibitor proteins compared to wounded control plants supplied with buffer alone. Pretreatment with RR and EB resulted in an 80–95% reduction in wound induced accumulation of proteinase inhibitors. Taken together, the effects of ionophores and Ca^{2+} transport antagonists strongly suggest that increases in cytosolic levels of free Ca^{2+} are a component of the signaling pathway leading to proteinase inhibitor synthesis.

3.3. Ca^{2+} transport antagonists differentially inhibit proteinase inhibitor induction by systemin and methyl jasmonate

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MJ) are powerful inducers of proteinase inhibitor synthesis [40]. Since systemin and MJ are potent inducers of the wound response, we wanted to determine if calcium transport antagonists would affect their ability to induce proteinase inhibitor synthesis. Fig. 3 shows that incubation of plants with RR prior to treatment with systemin or MJ had negligible effects on proteinase inhibitor synthesis. However, supplying plants with EB prior to treatment with systemin and MJ significantly reduced proteinase inhibitor synthesis compared to water or buffer controls.

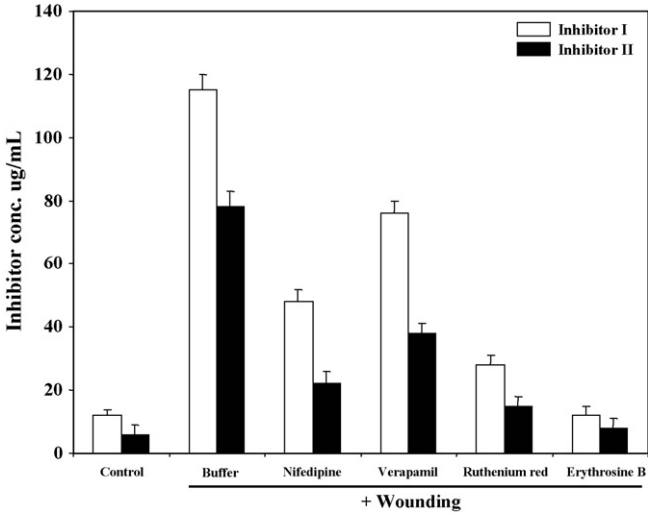

Fig. 2. Inhibition of proteinase inhibitor synthesis by calcium transport antagonists. Excised plants were supplied with test compounds at a concentration of 10 μM for 1 h prior to wounding. They were then transferred to water and incubated for 24 h. Control plants were supplied with buffer alone (Control) and not wounded or incubated in buffer for 1 h and subsequently wounded on the leaves (Buffer). After 24 h, the leaves were assayed for proteinase inhibitor accumulation. All solutions were supplied to plants in 10 mM phosphate buffer, pH 6.8. Data represent the mean ± S.D. of four independent experiments (six plants/treatment).

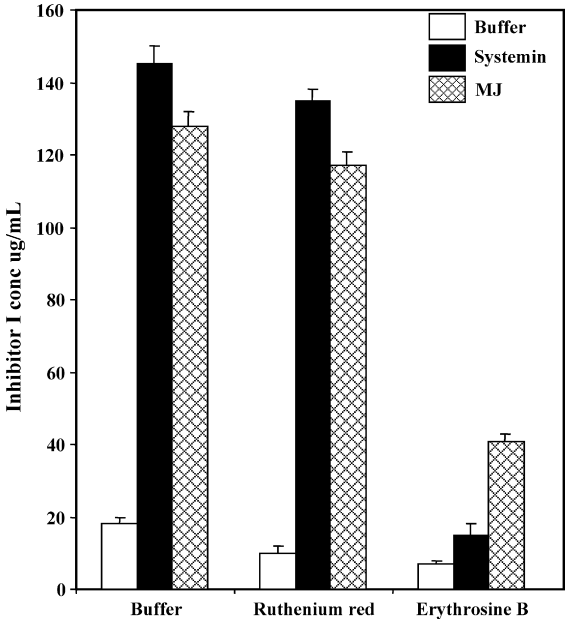

Fig. 3. Effects of calcium transport antagonists on proteinase inhibitor accumulation induced by methyl jasmonate (MJ) or systemin. Excised plants were supplied with calcium function antagonists for 1 h prior to treatment with MJ (cross-hatched bars) or supplied with 2.5 pmol of systemin (shaded bars), then transferred to water and incubated for 24 h. Control plants were supplied with buffer alone or buffer supplemented with the test compounds with no further treatment (open bars) for 1 h and then transferred to water. After 24 h the leaves were assayed for proteinase inhibitor accumulation. Ruthenium red (10 μM); erythrosine (10 μM). All solutions were supplied to plants in 10 mM phosphate buffer, pH 6.8. Data represent the mean ± S.D. of a minimum of three independent experiments for each treatment (six plants/treatment).

3.4. Calcium chelators induce proteinase inhibitor synthesis

Ca^{2+} fluxes initiated by wounding might involve several different Ca^{2+} sources inside and outside the cell. Therefore, we investigated the effects of Ca^{2+} chelators on the wound response with respect to Ca^{2+} ions present in the extracellular matrix. Initially, we performed feeding assays with the Ca^{2+} chelator EGTA at 10 mM. EGTA treatment had little effect on the levels of proteinase inhibitors synthesized in response to wounding (data not shown). However EGTA, supplied to plants at 10 mM in the absence of wounding, induced the accumulation of proteinase inhibitors to roughly 80% the levels produced by wounding or treatment with systemin at 2.5 pmol (Fig. 4). The proteinase inhibitor inducing activity of EGTA was concentration-dependent. Another specific chelator of Ca^{2+}, BAPTA, also induced proteinase inhibitors in a concentration-dependent manner. We next investigated the effects of less specific chelators, such as EDTA and citric acid, and found that these compounds also induced proteinase inhibitors (Fig. 4).

It has been shown that watering the root system of tomato plants with high NaCl concentrations activates the wound response to high levels in intact tomato plants [41]. In order to determine if the induction of proteinase inhibitors by EGTA was due to osmotic stress in the leaf, we supplied tomato plants with increasing concentrations of NaCl through their cut stems, and monitored proteinase inhibitor induction. As shown in Fig. 5, negligible proteinase inhibitor accumulation was observed in tomato plants when supplied with increasing concentrations of NaCl. This indicated that NaCl administered through cut stems has different effects than NaCl supplied to the root system, and that the protein inhibitor synthesis induced by EGTA was not a result of osmotic or Na^+ (EGTA's counter ion) stress.

In order to determine what component(s) of the signaling cascade was being affected by the chelators, we examined the chelator-induced accumulation of proteinase inhibitors in transgenic antisense-*PS* plants, and the mutant plants *spr-1* and *def-1*. These plants are dramatically impaired in the systemic accumulation of proteinase inhibitors in response to wounding. The antisense-*PS* plant is impaired in the wound response due to the silencing of the prosystemin gene, resulting in very low prosystemin transcript levels [42]. The *spr-1* mutant is defective in a systemin-specific signaling step that couples systemin perception to activation of the octadecanoid pathway [43–44]. As shown in Fig. 6, wild type plants supplied with 10 mM EGTA, displayed high levels of Inh I, reaching 85% of the systemin elicited response, and as shown in Fig. 4. The antisense-*PS* plants showed a similar pattern of accumulation, but Inh I levels were approximately 60% of the wild type levels. Inh I levels in the *spr-1* mutants treated with EGTA were about 85% of the levels induced by EGTA in the wild type plants, whereas the *def-1* plants showed only low level induction of Inh I accumulation in response to EGTA. Notably, in the *def-1* plants, Inh I accumulation in response to EGTA was almost double the amount induced from the wound peptide systemin. Similar trends for all plants were observed for Inh II accumulation (data not shown). These results indicate that the EGTA effect is independent of systemin, but dependent on jasmonic acid signaling.

3.5. Divalent cations enhance the potency of systemin

The peptide systemin has been shown to be a powerful inducer of the wound response [6]. During mechanical wounding, cells are disrupted releasing their contents to the apoplastic spaces of the leaf. Ca^{2+} and magnesium (Mg^{2+}) ions from intracellular stores and systemin are released and may act together to induce the wound response. However, when supplying systemin to plants through the cut stem very little cellular content is released to interact with systemin in the extracellular matrix. Therefore, we sought to determine whether elevated calcium and magnesium ion concentrations would have an effect on systemin activity.

Systemin was supplied, through cut stems, at different concentrations to young tomato plants in the presence of water, 10 mM $CaCl_2$, or 10 mM $MgSO_4$. As shown in Fig. 7A and B, the presence of these divalent cations decreased the concentration of systemin necessary to induce maximal proteinase inhibitor accumulation by 100-fold from 2.5 pmol to 25 fmol per plant. In the presence of 10 mM $MgSO_4$, 25 fmol of systemin produced a similar level of Inh I (Fig. 7A), and a 90% increase in the level of Inh II (Fig. 7B) accumulation, as

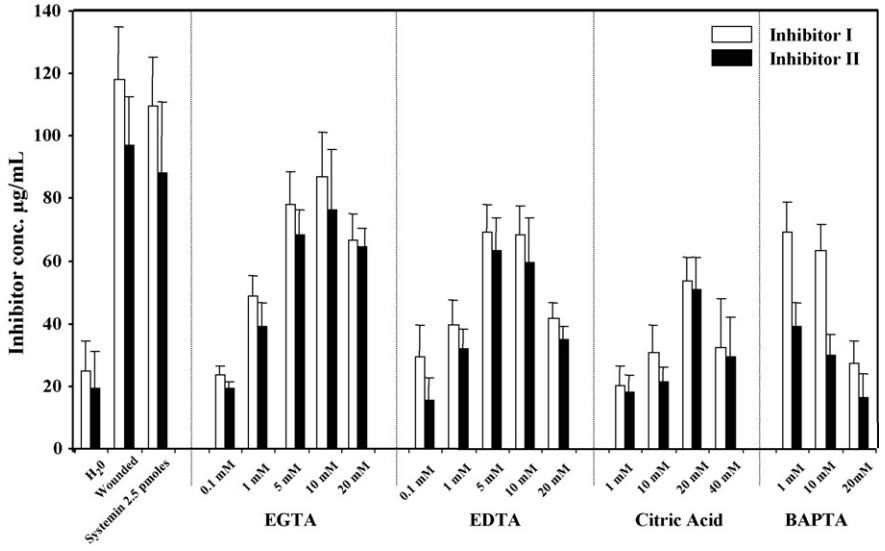

Fig. 4. Accumulation of proteinase inhibitors in response to chelators. Tomato seedlings were excised and supplied for 1.5 h through their cut stems with aequous solutions of chelators at the indicated concentrations. Proteinase inhibitor accumulation in leaves was assayed 24 h after treatment. Data represent the mean ± S.D. of one representative experiment ($n \geq 6$ plants/treatment). Similar results were obtained in at least five independent experiments.

compared to the levels of inhibitors produced from 2.5 pmol of systemin in water alone. In the presence of the divalent cations, systemin supplied at 2.5 pmol showed very little enhancement in its proteinase inhibitor inducing activity (Fig. 7A and B).

To further investigate Mg^{2+} and Ca^{2+} enhancement of systemin activity, we varied the concentrations of the cations in the presence of 25 fmol of systemin. As shown in Fig. 8, $CaCl_2$ and $MgSO_4$ solutions alone in absence of systemin did not induce the accumulation of proteinase inhibitors. However, supplementation of 25 fmol of systemin with as low as 0.1 mM of either $CaCl_2$ or $MgSO_4$ enhanced systemin activity and roughly doubled the amount of proteinase inhibitor accumulation in leaves, when compared to the same amount of systemin supplied only in water. The maximal systemin-enhancing effect for $CaCl_2$ was observed at a 10 mM concentration and decreased at 20 mM, while the effect of $MgSO_4$ was still increasing at a concentration of 20 mM.

Since systemin activity was significantly enhanced in the presence of $CaCl_2$ and $MgSO_4$, we wanted to determine if other divalent cations would also exhibit similar effects. Tomato seedlings were supplied with either 10 mM salt solutions alone, or the salt solution in the presence of 25 fmol of systemin. As shown in Fig. 9, none of the divalent cation solutions in absence of systemin induced the synthesis of Inh I significantly above background levels. As indicated above, Mg^{2+} and Ca^{2+} ions displayed strong systemin enhancing activity, whereas manganese (Mn^{2+}), in the form of manganese chloride, had less of an enhancing effect on systemin activity. The remaining divalent cations examined showed no enhancing effects. Interestingly, the counter ion associated with the Mg^{2+} ion appears to influence its enhancing effect, with sulfate displaying a more potent effect than the chloride ion. Similar trends were observed for Inh II (data not shown).

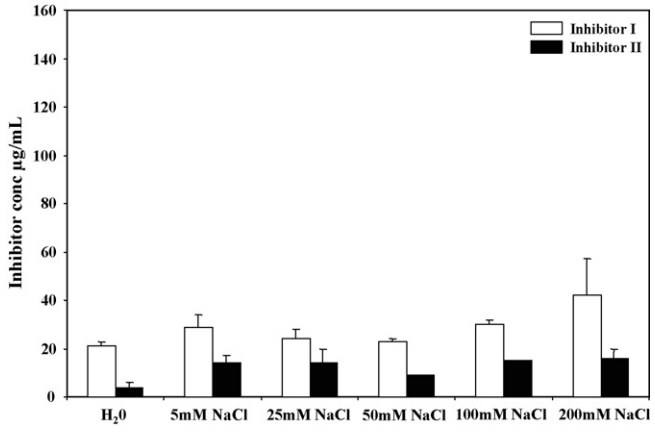

Fig. 5. Proteinase inhibitor accumulation in response to increasing concentrations of NaCl. 15 day-old tomato seedlings were supplied through their cut stems with different concentrations of NaCl as indicated, or water (H_2O) for 1.5 h. The bars represent the levels of proteinase inhibitor accumulation in unwounded leaves 24 h after treatment. Data represent the mean ± S.D. of a minimum of three independent experiments showing similar results (six plants/treatment).

4. Discussion

Homeostatic Ca^{2+} concentration gradients are established, and rigorously maintained, across biological membranes. In plant cells the concentration of free Ca^{2+} is, on average, 1000-fold higher outside the cell than in the cytosol. Disruption of this concentration gradient by treatment with Ca^{2+}-specific ionophores or inhibitors of membrane-bound Ca^{2+} channels can lead to inappropriate activation, or inhibition, of Ca^{2+}-regulated processes. We designed experiments aimed at altering intracellular levels of free Ca^{2+}, and then examining the effects of the altered Ca^{2+} levels on activation of the wound response cascade. Treatment of young tomato plants with the Ca^{2+} ionophores, ionomycin and A23187, induced the accumulation of proteinase inhibitor proteins to levels similar

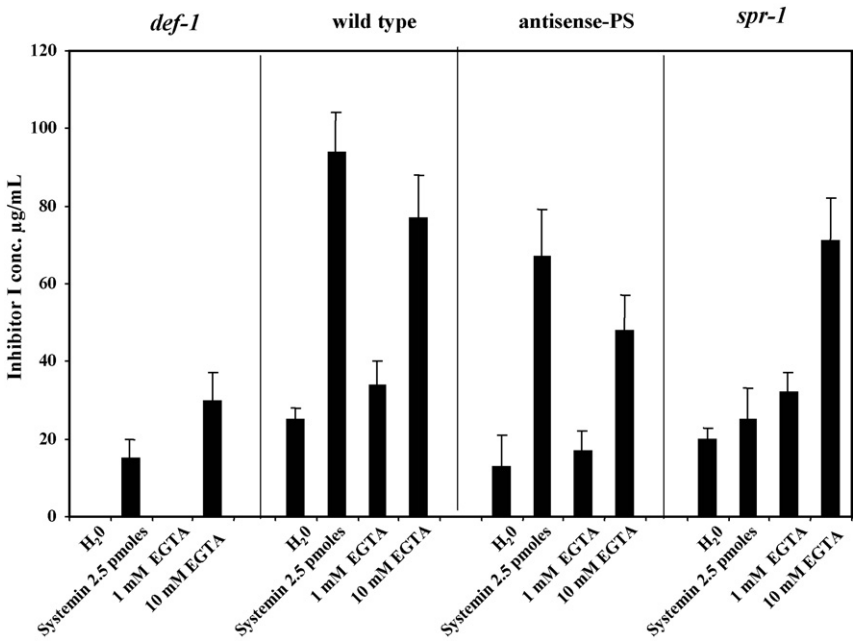

Fig. 6. The accumulation of proteinase inhibitor I protein in response to EGTA in antisense-*PS*, *spr-1* and *def-1* plants. 15 day-old tomato seedlings of wild-type, antisense-*PS*, *spr-1* and *def-1* mutant plants were excised and supplied through their cut stems with EGTA or systemin at the indicated concentrations in water for 1.5 h. The bars represent the levels of Inh I accumulation in the unwounded leaves 24 h after treatment. Data represent the mean ± S.D. of a minimum of three independent experiments showing similar results (six plants per treatment).

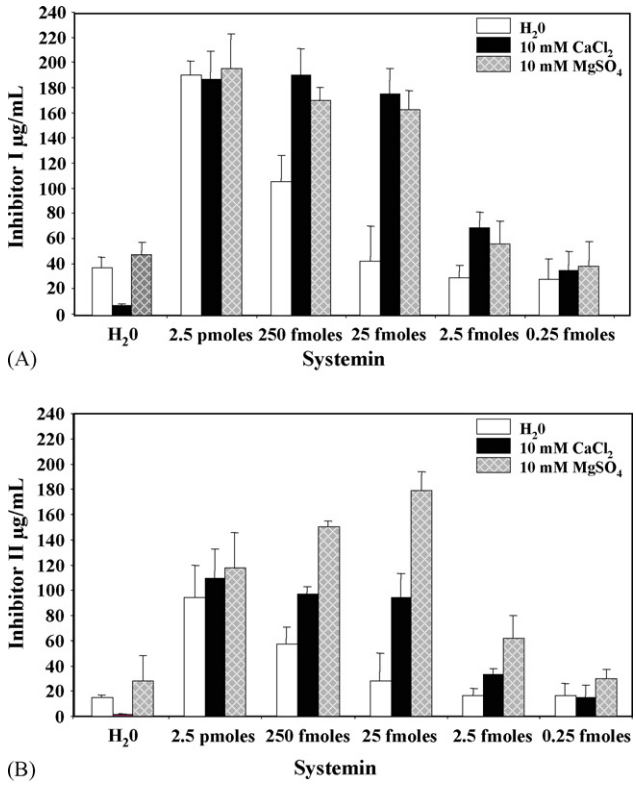

Fig. 7. Systemin activity is enhanced by the presence of calcium and magnesium cations. Tomato seedlings were supplied through their cut stems with decreasing amounts of systemin in the presence of water (open bars), 10 mM $CaCl_2$ (shaded bars) or 10 mM $MgSO_4$ (cross-hatched bars) for 1.5 h [pmol = pmol/plant; fmol = fmol/plant]. Proteinase inhibitor I (7A) and inhibitor II (7B) accumulation in leaves was measured 24 h after treatment. Data represent the mean ± S.D. of a typical experiment. Similar results were obtained in at least four independent experiments (six plants/experiment).

to those measured after wounding (Fig. 1A). This effect was not observed in the tomato *def-1* mutant (Fig. 1B), indicating that Ca^{2+} fluxes associated with the accumulation of proteinase inhibitors activate the octadecanoid pathway resulting in wound response gene activation. Ionophores generally are non-specific and interact with all biological membranes (e.g., plasma membrane and internal organelles), therefore ionophore-mediated increases in the levels of cytosolic Ca^{2+} most likely represent combined contributions from both extracellular and intracellular Ca^{2+} stores.

The Ca^{2+} channel blockers verapamil (Ver) and nifedipine (Nif) have been shown to block voltage-regulated L-type Ca^{2+} channels in animal cells by binding to a subunit of the Ca^{2+} channel complex [45,46]. These compounds were assayed for their effects on proteinase inhibitor accumulation in leaves of tomato plants. Verapamil has been shown to inhibit Ca^{2+} channels located in the tonoplast of plant root cells [47], and to reduce the influx of Ca^{2+} at the edge of the bite zone during feeding of *Spodoptera littoralis* on lima bean leaves [18]. When Nif and Ver were supplied to plants prior to wounding, we observed a strong reduction in the accumulated levels of proteinase inhibitor proteins when compared to wounding in the absence of the antagonists (Fig. 2). These results suggest that L-type Ca^{2+} channels play a role in the normal wound response.

To further investigate the role of Ca^{2+} channels in the wound response we investigated the effects of ruthenium red (RR), which is generally thought to inhibit plasma membrane Ca^{2+} channels, as well as intracellular endomembrane Ca^{2+} channels [47–50]. Ruthenium Red in potato plants has been shown to block induction of jasmonic acid biosynthesis and Inh II gene expression associated with heat-activated action potentials

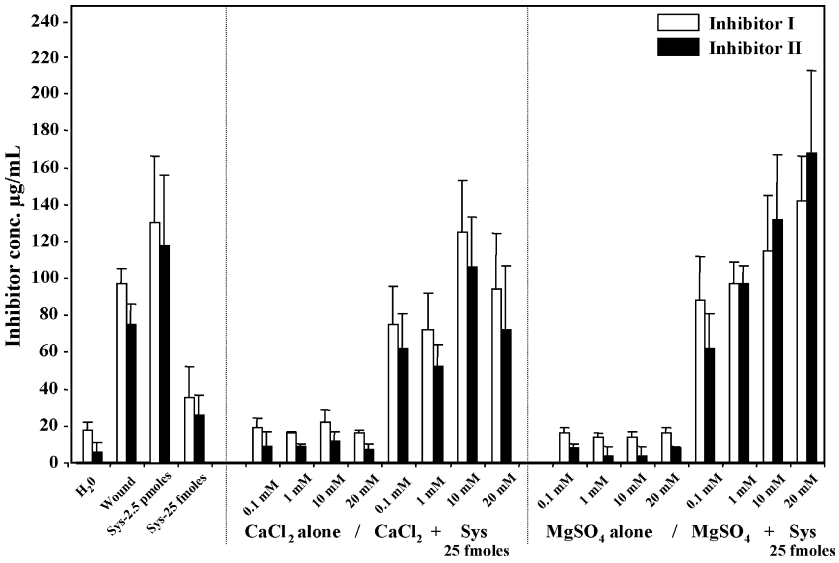

Fig. 8. The enhancement of systemin activity calcium and magnesium cations is concentation dependent. Tomato seedlings were supplied through their cut stems with systemin or water with different concentrations of $CaCl_2$ or $MgSO_4$ as indicated for 1.5 h. Proteinase inhibitor accumulation in leaves was measured 24 h after treatment [Sys-2.5 pmol = 2.5 pmol systemin/plant; Sys-25 fmol = 25 fmol systemin/plant]. Data represent the mean ± S.D. of at least four independent experiments (six plants/treatment).

[51]. In addition, RR has also been shown to interfere with Ca^{2+} release from vacuoles [52–53]. Fig. 2 shows that RR supplied to plant cells before wounding severely inhibited the induction of proteinase inhibitors in the wounded leaves. These experiments addressed the channel-mediated role of Ca^{2+} influxes into the cytosol, and confirmed that Ca^{2+} channels play a role in mediating wound response activation.

We then looked at the role of Ca^{2+}-ATPases involved in transporting Ca^{2+} from the cytosol back into the organelles and the apoplast. EB has been shown to inhibit both plasma membrane (PM) and endoplasmic reticulum (ER) associated Ca^{2+}-ATPases in plants [54–56]. Similar to RR, pre-incubation of tomato plants with EB completely blocked the synthesis of proteinase inhibitors in response to wounding. However, EB and RR exhibited different effects on proteinase inhibitor induction in response to MJ exposure or treatment with systemin. Pre-treatment of tomato plants with EB severely inhibited their ability to accumulate proteinase inhibitors in response to systemin or MJ, whereas RR showed no inhibitory effects under similar conditions (Fig. 3). Overall the data show that inhibition of Ca^{2+}-ATPases affects the response to wounding and systemin in a similar way.

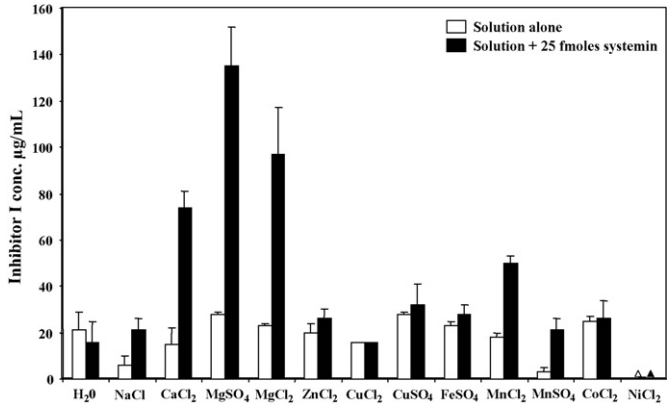

Fig. 9. The presence of specific divalent cations increases the potency of systemin. Fifteen day-old tomato seedlings were supplied through their cut stems with 10 mM solutions of divalent salts in water (open bars) or in the presence of 25 fmol of systemin (shaded bars): NaCl was supplied at 20 mM concentration for 1.5 h. The bars represent the levels of proteinase inhibitor I accumulation 24 h after treatment. Data represent the mean ± S.D. of a minimum of three independent experiments showing similar results (six plants/treatment).

Ca^{2+} fluxes are often one of the earliest signaling events after perception of an inducing signal. Consistent with this, we found that Ca^{2+} fluxes associated with effects of ionophores function proximal to JA biosynthesis (data not shown). Therefore, it was surprising to see that EB strongly reduced the response to MJ. Differential effects on gene induction or inhibition by various Ca^{2+} antagonists are not uncommon. A variety of effects by different antagonists were observed in studying the Ca^{2+} requirements of ethylene-dependent response in mung bean and pea tissues [57–59]. However, the overall effects observed while using the various Ca^{2+} antagonists clearly point to the interplay of ion channels, pumps, and Ca^{2+} pools during activation of the wound response by mechanical wounding and application of systemin. Taken together, the results described here, which demonstrated proteinase inhibitor induction by Ca^{2+} ionophores (Fig. 1A and B) and their inhibition by Ca^{2+} transport antagonists (Figs. 2 and 3), are consistent with a Ca^{2+}-dependent defense signaling pathway.

Chelators bind Ca^{2+} ions making them unavailable in solutions. We expected to see an inhibitory effect of Ca^{2+}-chelators on the wound response. Instead we found that Ca^{2+}-chelators induced proteinase inhibitor synthesis in the absence of wounding (Fig. 4). In order to determine what component(s) of the signaling cascade was being affected by the chelators, we investigated the effects of EGTA in wound response

mutants in tomato. The *def 1* mutant is impaired in the octadecanoid pathway and thus compromised in its ability to accumulate JA in response to wounding and elicitors [39]. When the *def-1* mutant was supplied with EGTA it exhibited a severe decrease in the accumulation of the Inh II protein as compared to similarly treated wild type plants (Fig. 6). This indicates that an intact octadecanoid pathway is necessary for EGTA-induced accumulation of the wound-inducible proteinase inhibitors. The low levels of Inh II protein in *def-1*, 10–20% of wild type levels are most likely due to the ''leaky'' nature of the mutant [39].

Previously it has been reported that when 10 mM EGTA was applied to tomato leaves, in which their epidermis had been removed, the presence of the chelator did not significantly affect the systemin induced increase in cytosolic Ca^{2+} [17]. However, in *L. peruvianum* cell cultures the extracellular Ca^{2+} chelator BAPTA was found to suppress medium alkalinization in response to systemin [13]. To determine whether systemin action was necessary for EGTA-induced accumulation of inhibitor proteins, EGTA was supplied to antisense-*PS* and *spr-1* mutant tomato plants. Exposure of the *spr-1* mutant and antisense-*PS* tomato plants to EGTA resulted in a significant accumulation of Inh II. These results indicated that systemin was not necessary for EGTA-induced accumulation of the Inh II protein. It is unclear how these chelators are activating wound-related genes. Previous research has shown that EGTA did not disrupt increases in cytoplasmic Ca^{2+} levels in response to mechanical stimuli [60] or systemin [17]. Further research into the action of these chelators is needed and may provide greater insight into wound response activation, and calcium signaling processes in general.

As discussed above, Ca^{2+} antagonists have differential effects on systemin and wounding. One of the significant differences between wounding and treatment with systemin is that during the wounding process cellular components including divalent cations are released into the extracellular matrix, which may contribute to activation of the wound response. Intracellular contents are generally absent during supplementation of systemin to leaves via the cut stem or to suspension-cultured cells. In plant cells, intracellular Ca^{2+} concentrations can be as high as 10 mM in the vacuole and 1 mM in other intracellular compartments, whereas extracellular Ca^{2+} levels can exceed 1 mM [24]. Another divalent cation critical to the proper function of plant cells is magnesium (Mg^{2+}). The concentration of free cytoplasmic Mg^{2+} is on the order of 1 mM and the content of some leaf endodermal cells has been measured at values as high as 100 mM in storage compartments [61]. During wounding, cations, such as Ca^{2+} and Mg^{2+}, derived from intracellular stores may interact with systemin in the apoplast to induce a localized wound response. In order to elevate ion concentrations in the extracellular matrix, we supplied systemin to plants in the presence of different divalent cations. Surprisingly, the addition of Ca^{2+} and Mg^{2+} ions profoundly enhanced systemin's activity decreasing the concentration of systemin necessary to induce maximal proteinase inhibitor accumulation by 100-fold (Figs. 7 and 8). We tested a range of other divalent cations of which only Mn^{2+} increased systemin activity slightly (Fig. 9). Since the chloride and sulfate counter anions for Ca^{2+} and Mg^{2+} were also present in other inactive salts tested, the effect of $CaCl_2$ and $MgSO_4$ is clearly due to the cation.

The mechanism for the increased biological activity of systemin in the presence of Ca^{2+} and Mg^{2+} is unknown. Systemin has been shown to trigger an influx of extracellular Ca^{2+} via several different types of Ca^{2+} channels as well as cause the mobilization of internal Ca^{2+} stores (our data and Ref. [17]). These influxes could be enhanced due to the presence of increased extracellular concentration of Ca^{2+} ions after wounding. Furthermore it has been shown that in fava bean guard cells Mg^{2+} ions sensitize slow vacuolar channels to physiological levels of cytosolic Ca^{2+} that lead to slow vacuolar current activation [62]. The report by Pei et al. [62] also indicate synergistic effects between Ca^{2+} and Mg^{2+} ions, and that Mg^{2+} can play an important role in the regulation of vacuolar ion channels. Mg^{2+} ions could play a similar role to enhance the activity of some ion channels as a result of their increased concentration outside the cell after wounding.

How physiologically relevant it is to supply concentrations of Ca^{2+} and Mg^{2+} ions as high as 10 mM to plants? Although ion concentrations of 10 mM may not be present throughout the whole leaf, when cells are disrupted upon wounding release of intracellular stores of Ca^{2+} and Mg^{2+} ions may create microenvironments of exceptionally high local concentrations. In these microenvironments, systemin may interact synergistically with these ions when binding to its receptor, or cause activation and enhancement of Ca^{2+} channels being used by the localized increases in divalent cations. However, it is impossible to determine the actual ion concentrations at the target sites. When supplying plants with Ca^{2+} and Mg^{2+} ions through their cut stems, the effective concentrations of the divalent cations may not actually be as high as 10 mM when reaching target tissues (Figs. 7–9). Plants were only exposed to these solutions for 90 min, and were subsequently chased with water for the next 24 h. Notwithstanding the potential limitations noted above, the work described here clearly indicates that the presence of the divalent Ca^{2+} and Mg^{2+} cations have a highly significant effect on the biological activity of systemin.

Ca^{2+} appears to be a ubiquitous signal in all tissues of plants where oscillations of cytosolic Ca^{2+} levels in response to environmental or developmental stimuli have been observed. Specificity of the plant's response to these oscillations still remains unclear. Potential targets of the Ca^{2+} signal are Ca^{2+} binding proteins such as calmodulin and Ca^{2+}-dependent kinases. The research described here is the first report that directly correlates calcium fluxes and wound gene activation. In addition to Ca^{2+}fluxes, our data demonstrated that elevated concentrations of calcium or magnesium ions in the apoplast significantly enhanced the biological activity of systemin. It is now clear from the work described here, and that of others, that Ca^{2+} does play a significant role in the activation of the wound response. The specific components or proteins of the wound signaling pathway that interact with these ions will need to be addressed in future work.

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References

[1] D.R. Bergey, G.A. Howe, C.A. Ryan, Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 12053–12058.

[2] G.A. Howe, Jasmonates as signals in the wound response, J. Plant Growth Regul. 23 (2004) 223–237.

[3] A.L. Schilmiller, G.A. Howe, Systemic signaling in the wound response, Curr. Opin. Plant Biol. 8 (2005) 369–377.

[4] J.S. Graham, G. Hall, G. Pearce, C.A. Ryan, Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants, Planta 169 (1986) 399–405.

[5] C.A. Ryan, Proteinase inhibitor genes in plants: genes for improving defenses against insects and pathogens, Annu. Rev. Phytopathol. 28 (1990) 425–449.

[6] G. Pearce, D. Strydom, S. Johnson, C.A. Ryan, A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins, Science 253 (1991) 895–898.

[7] G. Pearce, D. Moura, J. Stratmann, C.A. Ryan, Production of multiple plant hormones from a single polyprotein precursor, Nature 411 (2001) 817–820.

[8] C.A. Ryan, G. Pearce, Systemins:, A functionally defined family of peptide signals that regulate defensive genes in Solanaceae species, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 14577–14580.

[9] R.-G. María del Carmen, S.H. Carlos, S.H. Carla, M-G.N. Angélica, O.-A. Neftalí, J.P. Délano-Frier, The expression of the hydroxyproline-rich glycopeptide systemin precursor A in response to (a)biotic stress and elicitors is indicative of its role in the regulation of the wound response in tobacco (*Nicotiana tabacum* L.), Planta 222 (2005) 794–810.

[10] J.M. Scheer, C.A. Ryan, The systemin receptor SR160 from Lycopersicon peruvianum is a member of the LRR receptor kinase family, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 9585–9590.

[11] G. Felix, T. Boller, Systemin induces rapid ion fluxes and ethylene biosynthesis in *Lycopersicon peruvianum* cells, Plant J. 7 (1995) 381–389.

[12] C. Moyen, E. Johannes, Systemin transiently depolarizes the tomato mesophyll cell membrane and antagonizes fusicoccin-induced extracellular acidification of mesophyll tissue, Plant Cell Environ. 19 (1996) 464–470.

[13] A. Schaller, C. Oecking, Modulation of plasma membrane H+-ATPase activity differentlally activates wound and pathogen defense responses in tomato plants, Plant Cell 11 (1999) 263–272.

[14] M.L. Orozco-Cárdenas, J. Narváez-Vásquez, C.A. Ryan, Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate, Plant Cell 13 (2001) 179–191.

[15] J. Stratmann, C.A. Ryan, Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 11085–11089.

[16] S.R. Holley, R.D. Yalamanchili, D.S. Moura, C.A. Ryan, J.W. Stratmann, Convergence of signaling pathways induced by systemin, oligosaccharide elicitors, and ultraviolet-B radiation at the level of mitogen-activated protein kinases in *Lycopersicon peruvianum* suspension-cultured cells, Plant Physiol. 132 (2003) 1728–1738.

[17] C. Moyen, K.E. Hammond-Kosack, J. Jones, M.R. Knight, E. Johannes, Systemin triggers an increase in cytoplasmic calcium in tomato mesophyll cells: Ca2+mobilization from intra- and extracellular compartments, Plant Cell Environ. 21 (1998) 1101–1111.

[18] M. Maffei, S. Bossi, D. Spiteller, A. Mithöfer, W. Boland, Effects of feeding *Spodoptera littoralis* on lima bean leaves. I. membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components, Plant Physiol. 134 (2004) 1752–1762.

[19] D.R. Bergey, C.A. Ryan, Wound and systemin-inducible calmodulin gene expression in tomato leaves, Plant Mol. Biol. 40 (1999) 815–823.

[20] S. Lee, S. Suh, S. Kim, R.C. Crain, J.M. Kwak, H.G. Nam, Y. Lee, Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants, Plant J. 12 (1997) 547–556.

[21] J. Narváez-Vásquez, J. Florin-Christensen, C.A. Ryan, Positional specificity of a phospholipase A activity induced by wounding, systemin, and oligosaccharide elicitors in tomato leaves, Plant Cell 11 (1999) 2249–2260.

[22] J. León, E. Rojo, J.J. Sánchez-Serrano, Wound signalling in plants, J. Exp. Bot. 52 (2001) 1–9.

[23] D. Sanders, C. Brownlee, J.F. Harper, Communicating with calcium, Plant Cell 11 (1999) 691–706.

[24] B.B. Buchanan, W. Gruissem, R.L. Jones, Biochemistry & Molecular Biology of Plants, first ed., American Society of Plant Physiologists, Rockville, MD, 2000.

[25] S.A.M.G. Scrase-Field, M.R. Knight, Calcium: just a chemical switch? Curr. Opin. Plant Biol. 6 (2003) 500–506.

[26] J. Sheen, Ca^{2+}-dependent protein kinases and stress signal transduction in plants, Science 274 (1996) 1900–1902.

[27] A.C. Harmon, M. Gribskov, J.F. Harper, CDPKs: A kinase for every Ca^{2+} signal? Trends Plant Sci. 5 (2000) 154–159.

[28] S.H. Cheng, M.R. Willmann, H.C. Chen, J. Sheen, Calcium signaling through protein kinases: The Arabidopsis calcium-dependent protein kinase gene family, Plant Physiol. 129 (2002) 469–485.

[29] T. Yang, B.W. Poovaiah, Calcium/calmodulin-mediated signal network in plants, Trends Plant Sci. 8 (2003) 505–512.

[30] J.M. Chico, M. Raices, M.T. Tellez-Inon, R.M. Ulloa, A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants, Plant Physiol. 128 (2002) 256–270.

[31] H. Yamakawa, I. Mitsuhara, N. Ito, S. Seo, H. Kamada, Y. Ohashi, Transcriptionally and post-transcriptionally regulated response of 13 calmodulin genes to tobacco mosaic virus-induced cell death and wounding in tobacco plant, Eur. J. Biochem. 268 (2001) 3916–3929.

[32] C.A. Ryan, Quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies, Anal. Biochem. 19 (1967) 434–440.

[33] H. Tokumitsu, T.R. Soderling, Requirements for calcium and calmodulin in the calmodulin kinase activation cascade, J. Biol. Chem. 271 (1996) 5617–5622.

[34] K.R. Chung, Involvement of calcium/calmodulin signaling in cercosporin toxin biosynthesis by *Cercospora nicotianae*, Appl. Environ. Microbiol. 69 (2003) 1187–1196.

[35] G. Sudha, G.A. Ravishankar, The role of calcium channels in anthocyanin production in callus cultures of *Daucus carota*, Plant Growth Regul. 40 (2003) 163–169.

[36] K.H. Uhm, Y.H. Lee, S. Kim, I.P. Ahn, Calcium/calmodulin-dependent signaling for prepenetration development in *Colletotrichum gloeosporioides*, Phytopathology 93 (2003) 82–87.
[37] M.J. Loeb, Altering the fate of stem cells from midgut of *Heliothis virescens*: the effect of calcium ions, Arch. Insect Biochem. Physiol. 59 (2005) 202–210.
[38] J. Lightner, G. Pearce, C.A. Ryan, J. Browse, Isolation of signaling mutants of tomato (*Lycopersicon esculentum*), Mol. Gen. Genet. 241 (1993) 595–601.
[39] G.A. Howe, J. Lightner, J. Browse, C.A. Ryan, An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack, Plant Cell 8 (1996) 2067–2077.
[40] E.E. Farmer, C.A. Ryan, Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors, Plant Cell 4 (1992) 129–134.
[41] J.E. Dombrowski, Salt stress activation of wound-related genes in tomato plants, Plant Physiol. 132 (2003) 2098–2107.
[42] B. McGurl, G. Pearce, M.L. Orozco-Cardenas, C.A. Ryan, Structure, expression, and antisense inhibition of the systemin precursor gene, Science 255 (1992) 1570–1573.
[43] G.A. Howe, C.A. Ryan, Suppressors of systemin signaling identify genes in tomato wound response pathway, Genetics 153 (1999) 1411–1421.
[44] G.I. Lee, G.A. Howe, The tomato mutant spr-1 is defective in systemin perception and the production of a systemic wound signal for defense gene expression, Plant J. 33 (2003) 567–576.
[45] K.P. Campbell, A.T. Leung, A.H. Sharp, The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel, Trends Neurosci. 11 (1988) 425–430.
[46] W.A. Catterall, M.J. Seagar, M. Takahashi, K. Nunoki, Molecular properties of voltage-sensitive calcium channels, Adv. Exp. Med. Biol. 255 (1989) 101–109.
[47] V.S. Anil, K.S. Rao, Calcium-mediated signal transduction in plants: a perspective on the role of Ca^{2+} and CDPKs during early plant development, J. Plant Physiol. 158 (2001) 1237–1256.
[48] J. Marshall, A. Corzo, R.A. Leigh, D. Sanders, Membrane potential-dependent calcium-transport in right-side-out plasma-membrane vesicles from *Zea mays* L. roots, Plant J. 5 (1994) 683–694.
[49] C.C. Subbaiah, D.S. Bush, M.M. Sachs, Elevation of cytosolic calcium precedes anoxic gene expression in maize suspension-cultured cells, Plant Cell 6 (1994) 1747–1762.
[50] M. Pineros, M. Tester, Calcium channels in higher plant cells: selectivity, regulation and pharmacology, J. Exp. Bot. 48 (1997) 551–577.
[51] J. Fisahn, O. Herde, L. Willmitzer, H. Pena-Cortes, Analysis of the transient increase in cytosolic Ca^{2+} during the action potential of higher *plants* with high temporal resolution: requirement of Ca2+ transients for induction of jasmonic acid biosynthesis and PINII gene expression, Plant Cell Physiol. 45 (2004) 456–459.
[52] G.J. Allen, S.R. Muir, D. Sanders, Release of Ca^{2+} from individual plant vacuoles by both InsP3 and cyclic ADP-ribose, Science 268 (1995) 735–737.
[53] G.J. Allen, D. Sanders, Vacuolar ion channels of higher plants, Adv. Bot. Res. 25 (1997) 218–252.
[54] M. Olbe, M. Sommarin, ATP-dependent Ca2+ transport in wheat root plasma membrane vesicles, Physiol. Plant 83 (1991) 535–543.
[55] P. Askerlund, D.E. Evans, Reconstitution and characterization of a calmodulin-stimulated Ca2+-pumping ATPase purified from *Brassica oleracea* L., Plant Physiol. 100 (1992) 1670–1681.
[56] L.J. Thomson, J.L. Hall, L.E. Williams, A study of the effect of inhibitors of the animal sarcoplasmic/endoplasmic reticulum-type calcium pumps on the primary Ca2+-ATPases of red beet, Plant Physiol. 104 (1994) 1295–1300.
[57] V. Raz, R. Fluhr, Calcium requirement for ethylene dependent responses, Plant Cell 4 (1992) 1123–1130.
[58] T. Jung, J.H. Lee, M.H. Cho, W.T. Kim, Induction of 1- aminocyclopropane-1-carboxylate oxidase mRNA by ethylene in mung bean roots: possible involvement of Ca^{2+} and phosphoinositides in ethylene signalling, Plant Cell Environ. 23 (2000) 205–213.
[59] L. Petruzzelli, M. Sturaro, D. Mainieri, G. Leubner-Metzger, Calcium requirement for ethylene-dependent responses involving 1-aminocyclopropane-1-carboxylic acid oxidase in radicle tissues of germinated pea seeds, Plant Cell Environ. 26 (2003) 661–671.
[60] A. Haley, A.J. Russell, N. Wood, A.C. Allan, M. Knight, A.K. Campbell, A.J. Trewavas, Effects of mechanical signaling on plant cell cytosolic calcium, Proc. Natl. Acad. Sci. 92 (1995) 4124–4128.
[61] R. Stelzer, H. Lehmann, D. Krammer, U. Luttge, X-Ray microprobe analysis of vacuoles of spruce needle mesophyll, endodermis and transfusion parenchyma cells at different seasons of the year, Botanica Acta 103 (1990) 415–423.
[62] Z.-M. Pei, J.M. Ward, J.I. Schroeder, Magnesium sensitizes slow vacuolar channels to physiological cytosolic calcium and inhibits fast vacuolar channels in fava bean guard cell vacuoles, Plant Physiol. 121 (1999) 977–986.